Preparation of Plasmid DNA

Step 1: Transformation of $CaCl_2$-competent E.coli cells

Introduction

Transformation of the your DNA plasmid

Components

• CaCl2-competent *E. coli* cells;
• SOC medium;
• DNA plasmid.
• Agar plates

Control

Water (a negative control)

Method

1. Turn on a water bath or heating block to 42°C.
2. Thaw competent cells on ice for 15 min.
3. Add 10 µL of ligation reaction mixture or controls above to 100 µL of competent cells.
4. Incubate for 30 min on ice.
5. Heat shock for 70 s at 42°C.
6. Incubate for 2 min on ice.
7. Add 400 µL of SOC media (pre-heated to 37°C).
8. Incubate for 1.5 hr at 37°C, with occasional gentle mixing by inversion of the tubes.
9. Add 100µL into an agar plate containing the appropriate antibiotic and spread it with a spreader.
10. Put the plates at 37°C overnight (14-16 hours).

Step 2: The liquid culture of a single bacterial colony

Introduction

To guarantee the resistant to the antibiotic of a bacterial clonal and obtain a certain amount of bacterial for the extraction of plasmid.

Components

• LB medium
• Antibiotic

Method

1.Pick out a single clony using a pipette.
2. xx the tip into the tube which contains 5ml LB liquid medium with appropriate antibiotic
3.Incubate for 12-14 hours at 37°C in a constant temperature shaker.

Step 3: Plasmid extraction

Introduction

To prepared plasmid DNA from bacterial colonies after 16-18 hours of growth in a liquid culture.

Kit

Omega EZNA plasmid mini kit I

Control

1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1- 5 mL LB medium containing the appropriate selective antibiotic. Incubate for 12-16 hours at 37°C with vigorous shaking (~ 300 rpm). Use a 10-20 mL culture tube or a flask with a volume of at least 4 times the volume of the culture. It is strongly recommended that an endA negative strain of E. coli be used for routine plasmid isolation. Examples of such strains include DH5a® and JM109®.
2. Centrifuge at 10,000 g for 1 minute at room temperature. Decant or aspirate and discard the culture media.
3. Add 250 µL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.
Note: RNase A must be added to Solution I before use. Add 250 µL Solution II. Invert and gently rotate the tube several times to obtain a clear lysate. A 2-3 minute incubation may be necessary.
4. Add 350 µL Solution III. Immediately invert several times until a flocculent white precipitate forms.
Note: It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.
5. Centrifuge at maximum speed (≥13,000 x g) for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.
6. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.
7. Transfer the cleared supernatant from Step 5 by CAREFULLY aspirating it into the HiBind® DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the HiBind® DNA Mini Column.
8. Centrifuge at 10000g speed for 1 minute.
9. Discard the filtrate and reuse the collection tube.
10. Add 500 µL HB Buffer.
11. Centrifuge at 10000g speed for 1 min
12. Discard the filtrate and reuse collection tube.
13. Add 700 µL DNA Wash Buffer.
14. Centrifuge at 10000g for 1 minute.
15. Discard the filtrate and reuse the collection tube.
Optional: Repeat Steps 13-15 for a second DNA Wash Buffer wash step.
16. Centrifuge the empty HiBind® DNA Mini Column for2 min at 13000g to dry the column matrix.
Note: It is important to dry the HiBind® DNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.
17. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.
18. Add 30-50 µL Elution Buffer or sterile deionized water directly to the center of the column membrane.
Note: The efficiency of eluting DNA from the HiBind® DNA Mini Column is dependent on pH. If using sterile deionized water, make sure that the pH is around

8.5.
19. Let sit at room temperature for 5 minute.
20. Centrifuge at 13000g for 1 minute.

Restriction Digest

Introduction

Transformation of the your DNA plasmid

Components

• dH2O
• DNA plasmid (Part A: J23101, J23106, J23117; Part B:I13504)
• Cutsmart buffer
• Restriction Enzymes: SpeI, XbaI, PstI,

Method

Step 1: Digestion

1. Keep all enzymes and buffers used on ice.
2. Thaw Cutsmart buffer and Restriction Enzymes on ice.
3. Add 1µg of DNA to the appropriately labelled tube.
4. Pipet 5ul of Cutsmart buffer to each tube.
5. In the Part A tube: Add 0.5ul of PstI and 0.5ul of SpeI to each tube.
6. In the Part B tube: Add 0.5ul of XbaI, and 0.5ul of PstI.
7. Add distilled water to the tubes for a total volume of 50ul in each tube.

Mix well by pipetting slowly up and down. Spin the samples briefly to collect all of the mixture to the bottom of the tube.

8. Incubate the restriction digests at 37°C for 30 minutes
9. Run a portion of the digest on a gel (8ul, 100ng)
10. Check that both plasmid backbone and part length are accurate.

Step 2: Gel extraction (To obtain the specific DNA segment)

Kit

The E.Z.N.A.® Gel Extraction Kit

1. Perform agarose gel electrophoresis to fractionate DN A fragments.
2. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean scalpel.
3. Determine the approximate volume of the gel slice by weighing it in a clean 1.5 ml microfuge tube. Assuming a density of 1 g/ml of gel, the volume of gel is derived as follows: A gel slice of m ass 0.3 g will have a volume of 0.3 ml. Add equal volume of Binding Buffer (XP2). Incubate the mixture at 55oC- 60oC for 7 min or until the gel has completely melted. Mix by shaking or inverting the tube every 2-3 minutes. Centrifuge the tube briefly to collect all the liquid to the bottom of the tube.

Note: For DNA fragment less than 500bp, add 1 sample volume of isopropanol after the addition of Binding Buffer (XP2).

Important: Monitor the pH of the G el/Binding Buffer mixture after the gel completely dissolves. DNA yield will significantly decrease when pH > 8.0. If the color of the mixture become orange or red, Add 5 :l of 5M sodium acetate, pH 5.2, to bring the pH down. After this adjustment, the color of the gel/Binding

Buffer mixture should be light yellow.

4. Apply up to 700 :l of the DN A/agarose solution to a HiBind ® DN A spincolumn assembled in a clean 2 ml collection tube (provided) and centrifuge in a microcentrifuge at 8,000-1 0, 00 0 x g for 1 min at room temperature. Discard the liquid. Re-use the collection tube in Steps 5-8. For volumes greater than 700:l, load the column and centrifuge successively, 700 :l at a time. Each HiBind ® spin-column has a total capacity of 25-30 g DNA.
5. Discard liquid and add 300: l Binding Buffer. Centrifuge at 10,000 x g for 1 minute.
6. Add 700:l of S PW Buffer diluted with absolute ethanol into the column and wait 2-3 minutes. Centrifuge at 10,000 x g for 1 min at room temperature to wash the sample.
7. **Optional**: Discard liquid and repeat step 6 with another 700:l SPW Buffer.

Note: Perform this second wash step for any salt sensitive downstream applications.

8. Discard liquid and, re-using the collection tube, centrifuge the empty column for 1 min at 10,000 x g to dry the column matrix. **This drying step is critical for good DNA yields.**
9. Place column into a clean 1.5 ml microcentrifuge tube (not provided). Add 30-50 :l (depending on desired concentration of final product) Elution Buffer (or sterile deionized water) directly to the center of the column matrix, then incubate for 1 minute. Centrifuge 1 min at 10, 000 x g to elute D NA. This represents approximately 70% of bound D NA. An optional second elution will yield any residual DNA, though at a lower concentration.

Note: Efficiency of eluting DNA from column is dependent on pH. If eluting DNA with water, make sure that the pH is around 8.0.

10. Yield and quality of DN A: determine the absorbance of an appropriate dilution of the sample at 260 nm and then at 280 nm. The DNA concentration is calculated as follows:
DNA concentration = Absorbance$_{260}$ × 50 × (Dilution Factor): g/ml
Fragments greater than 500 bp in length can routinely be purified at >80% yield. Bands ranging from 50 bp to 500 b p give yields of 55%-80%. The ratio of (absorbance260) / (absorbance280) is an indication of nucleic acid purity. A value greater than 1.8 indicates > 90% nucleic acid. Alternatively, yield (as well as quality) can sometimes best be determined by agarose gel/ethidium bromide electrophoresis.

Ligation

Introduction

To obtain the target DNA plasmid

Components

• DNA segment
• T4 DNA ligase buffer
• T4 DNA ligase
• dH2O

Method

1. Add 10ul of gel extraction DNA segment
2. Add 1 ul T4 DNA ligase buffer.
5. Add 0.5 ul T4 DNA ligase
6. Add water to 10 ul
7. Ligate 22°C for 30 min
8. Transform with 1-2 ul of product.

Clony PCR

Introduction

To prepare DNA samples from several colonies to screen for the desired mutant by sequencing.

Components

• A plate with re-streaked, single colonies;
• ddH2O
• Taq master from Gene star company
• F primer
• R primer

Method

1.Add 1ul F primer and 1ul R primer into a PCR tube
2.Add xul taq master
3.Pick out some bacterial into the PCR mix using pipette
4.Program the PCR machine as follow:

94°	00:05:00	1x
94°	00:00:30	30x
58°	00:00:30	
72°	00:00:30	
72°	00:07:00	1x
4°	∞	1x

5.Run a electrophoresis to identify the DNA plasmid.